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In Situ Detection of Basic Fibroblast Growth Factor by Highly Specific Antibodies

Klaus Schulze-Osthoff,* Werner Risau,‡ Ekkehard Vollmer,† and Clemens Sorg*

From the Institute of Experimental Dermatology and the Gerhard-Domagk Institute of Pathology,† University of Münster, Münster; and the Max-Planck-Institute of Psychiatry,† Martinsried, West Germany*

Basic fibroblast growth factor (bFGF) is thought to be of major importance for fibrosis and angiogenesis. Despite intensive studies dealing with the biochemistry and multiple biologic effects of bFGF, the cellular distribution is virtually unknown. Therefore, using the indirect immunoperoxidase technique, we examined the effect of bFGF on a large pattern of normal, inflammatory, and tumorous human tissues. Staining was performed on cryostat sections with a highly specific affinity-purified antiserum. In normal tissues, especially those of the thymus and placenta, mainly dendritic cells contained the growth factor. High levels of bFGF were also detected in basal cells and gland epithelial cells of skin biopsies. A conspicuous expression was observed in chronic inflammatory tissues corresponding to a generally pronounced proliferation of fibroblasts and endothelial cells in these situations. Tumors revealed a very heterogenous staining pattern. In some lesions, bFGF was predominantly present in infiltrating and endothelial cells. In several, neoplasms tumor cells exhibited an intensive staining. In some, especially vascular tumors, bFGF could not be aetected. From the staining results it is concluded that angiogenesis is not simply controlled by the presence of bFGF but is mediated by a balance of several angiogenic inducers and inhibitors. (Am J Pathol 1990, 137:85–92)

Fibroblast growth factors (FGFs), originally identified based on their mitogenicity for fibroblasts by now have been shown to possess profound effects on various cell types influencing either their proliferation, differentiation, or other functions (for review see refs. 1–4). Fibroblast growth factors are a family of polypeptides, with a molecular range of 15 to 18.5 kd, that can be classified into two groups: acidic and basic prototypes. Both mediators are closely related with respect to their primary structure and biologic activities, but they differ in their relative potency, with bFGF 30 to 100 times more potent than acidic FGF.[2] While acidic FGF is generally present in neural tissues and, furthermore, has been found in heart and kidney, bFGF seems to be more widespread. Due to its high affinity for heparin, bFGF could be easily purified from a variety of tissues, including placenta, prostate, thymus, kidney, corpus luteum, adrenal gland, retina, brain, and pituitary gland.[1–3] In addition to its mitogenic effects, bFGF increases the production of collagenases and plasminogen activator,[5,6] which are thought to be critical events in tissue remodeling. Of particular importance is the observation that bFGF not only stimulates endothelial cell proliferation *in vitro* but is also a potent inducer of blood vessel growth *in vivo*, a process called angiogenesis. Furthermore, when implanted in sponges in rats, bFGF has been shown to induce formation of highly vascularized granulation tissues.[7]

While bFGF is very well characterized biochemically, little is known about its physiologic relevance and the cell type expressing bFGF. Investigations about the cellular distribution are restricted to cultured cells in which several normal and tumor-derived cell lines, as well as macrophages, were found as a source of the mediator.[8–13] However, because there is a great plasticity and rapid functional change in cultured cells, cellular behavior *in vitro* may not necessarily correlate with *in vivo* function. To understand the role of growth factors in *in vivo* conditions, *in situ* analysis is required. Therefore it is the aim of this study to investigate the presence of bFGF in normal, inflammatory, and tumor tissues using the indirect immunoperoxidase technique.

Materials and Methods

Antibodies

Polyclonal antibodies against recombinant human bFGF[14] were raised in rabbits by multiple intradermal and

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Address reprint requests to Klaus Schulze-Osthoff, PhD, Institute of Experimental Dermatology, University of Münster, Von-Esmarch-Straße 56, 44 Münster, West Germany.

subcutaneous injections using 50 μg per immunization. They specifically detect bFGF but do not recognize acidic FGF in Western blot analysis at a 1000-fold higher dilution of the antisera. For immunohistochemical staining, the antiserum was purified by affinity chromatography: bFGF-specific antibodies were isolated by passage over bFGF coupled to sepharose.

Human Cells

Monocytes were obtained by leukapheresis[15] or were isolated from pooled buffy coats with Ficoll-Paque followed by hypotonic density gradient centrifugation in Percoll.[16] The cells were cultivated in Teflon bags[17] in McCoys 5A medium (Biochrom, Berlin, FRG), supplemented with 20% heat-inactivated serum from three donors at a density of 1×10^6 cells/ml in 7.5% oxygen-humidified atmosphere. Mononuclear cells and granulocytes were isolated from peripheral blood according to Boyum.[18] Lymphocytes were enriched from mononuclear cell fractions by Percoll density fractionation.[16] Platelets were obtained by differential centrifugation (2000*g*) of plasma supernatant after Ficoll-Paque treatment. For *in vitro* cell activation, cells were incubated for 24 hours with endotoxin (LPS) from *Escherichia coli*, serotype 055:B5 (25 μg/ml, Sigma, Deisenhofen, FRG). Cells were processed for cytospin preparations as described.[19]

Tissues

Biopsies of human tissues were snap frozen in liquid nitrogen and stored at −80°C. Five-micrometer thick frozen sections were prepared with a minotome (SLEE, Mainz, FRG) and fixed for 10 minutes in acetone.

Immunohistology

Cytospin preparations and sections were air dried and fixed for 5 minutes in acetone, washed with phosphate-buffered saline (PBS), and incubated in 0.4% sodium azide with 0.1% H_2O_2 in PBS for 15 minutes to inactivate endogenous peroxidases. After two washes in PBS, preincubation was carried out with 50% normal goat serum (NGS) in PBS for 30 minutes followed by incubation with anti-bFGF IgG (1, 5 μg/ml, diluted in 10% NGS). After 90 minutes, sections were washed three times in PBS and incubated for 45 minutes with goat-anti-rabbit IgG peroxidase conjugate (Dianova, Hamburg, FRG). After three additional washes, bound peroxidase was developed with 3-amino-4-ethyl-carbazole (AEC, Sigma) at pH 4.9 in acetate buffer plus 0.015% H_2O_2 for 15 minutes.

Sections were counterstained with Mayer's hemalaun (Merck, Darmstadt, FRG) for 1 minute and embedded in Aqua mount (BDH-Chemicals, Poole, GB).

Controls were performed either by (1) replacing the anti-bFGF IgG by normal rabbit IgG or (2) absorption of anti-bFGF IgG with a tenfold excess of recombinant human bFGF before the incubation.

Table 1. *Distribution of bFGF in Peripheral Blood Cells*

Cells	% positive cells (n = 3)
Platelets	0
Lymphocytes	0
Granulocytes	0
Monocytes d2	
Single donor, unstimulated	0
Single donor, LPS stimulated	50–80
Pooled buffy coat	15–60

Results

Distribution of bFGF in Peripheral Blood Cells

The presence of bFGF was tested on a large panel of peripheral blood cells of various donors (Table 1). Blood cells of single donors such as granulocytes, lymphocytes, and platelets never possess detectable amounts of bFGF. Positively stained monocytes, however, were found in pooled buffy coats of several donors (Figure 1a) or after stimulation of single donor cells with LPS.

Distribution of bFGF in Normal Tissues

When biopsies of normal tissues are examined, bFGF is found predominantly in resident cells of the myeloid lineage (Table 2).

In termed placenta, bFGF is detected in the villous mesenchym (Figure 1c). Positive cells are dendritic cells and Hofbauer cells, whereas no reaction is detected in the trophoblast. In spleen tissue bFGF mainly appears faintly throughout the red pulp and in some blood vessels, especially central arterioles of the white pulp (Figure 1d).

For samples of lymphoid tissues, thymus and unaffected lymph nodes were examined. While lymph nodes are generally negative or only contain few positively stained endothelial cells, pronounced bFGF expression is observed in scattered dendritic cells of the thymus cortex and medulla (Figure 1e). In skin biopsies, bFGF is rarely detected in histiocytes and endothelial cells but is strongly expressed by keratinocytes adjacent to the basal membranes (Figure 1f). In these biopsies hair follicles and sweat and sebaceous glands contain considerable amounts of bFGF. Samples of other normal tissues like liver, intestine, and coleum are consistently negative or, as in biopsies of kidney and lung, exhibit only a faint reactivity.

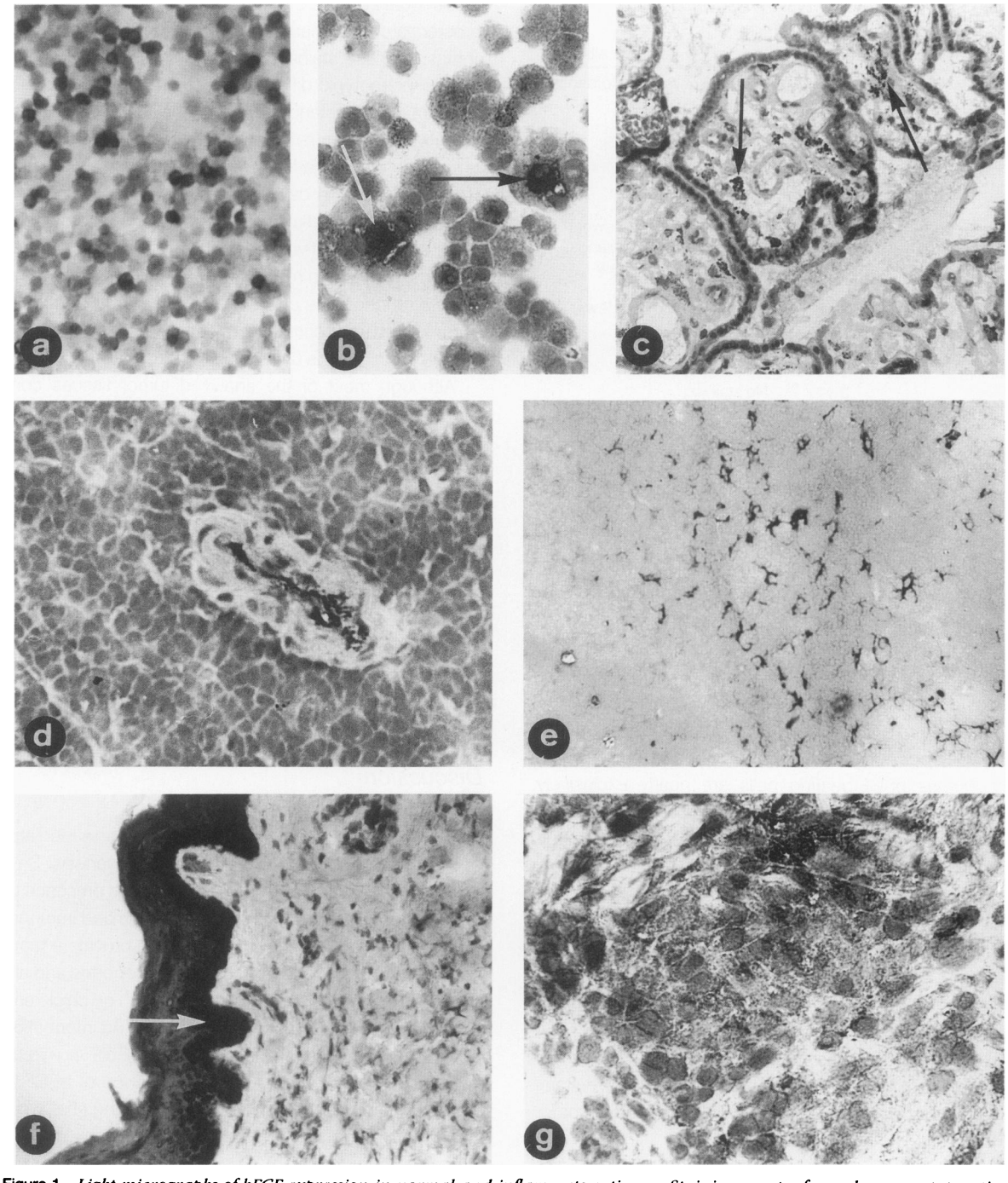

Figure 1. *Light micrographs of bFGF expression in normal and inflammatory tissues. Staining was performed on cryostat sections with a polyclonal antiserum to bFGF. Sections were counterstained with Mayer's hemalum.* **a:** *Monocytes (cytospin preparation of pooled buffy coats; ×600).* **b:** *Alveolar macrophages of sarcoidosis (cytospin preparation of a bronchoalveolar lavage; ×750).* **c:** *Placenta: bFGF appears within dendritic and Hofbauer cells (×600).* **d:** *Spleen: positively stained central arteriole of the white pulp (×600).* **e:** *Thymus: marked dendritic cells (×600).* **f:** *Normal skin: bFGF localizes within the basal keratinocytes of the epidermis (×600).* **g:** *Experimentally induced BCG granuloma: bFGF staining throughout the granuloma (×1900).*

Distribution of bFGF in Inflammatory and Tumor Tissues

Basic fibroblast growth factor is strongly expressed in pathologic situations correlating with intensive proliferation of fibroblasts and blood vessel formation (Table 3). Especially in conditions of chronic inflammation like rheumatoid arthritis, granuloma, and endometriosis, a pronounced staining is observed. Rheumatoid synovial tissues and BCG granuloma reveal considerable numbers of positively stained phagocyting cells as macrophages and synovial, respectively, epitheloid cells (Figure 1g). Alveolar macrophages of patients with sarcoidosis (isolated

Table 2. *Distribution of bFGF in Normal Tissues*

Tissues	No. of biopsies	bFGF positive cells
Placenta	3	Dendritic cells, few Hofbauer cells
Thymus	2	Dendritic cells
Lymph node	3	Single endothelial cells
Spleen	3	White pulp, few endothel. cells
Lung	2	Few single alveolar macrophages
Skin	4	Basal keratinocytes, sweat and sebaceous glands, few histocytes, few endothelial cells
Foreskin	2	Basal keratinocytes, few endothelial cells
Liver	2	No staining
Intestine	1	No staining
Colon	2	No staining

from bronchoalveolar lavages of three patients) expressed bFGF in up to 10% of the cells (Figure 1b). A striking phenomenon is the fact that biopsies of liver cirrhosis contain positively stained hepatocytes and sinusoidal cells in the fibrotic area, while no reaction can be detected in normal liver tissues. On the other hand, in biopsies of acute inflammations like cutaneous lesions, such as psoriasis, erythrodermia, urticaria, and parodontitis, staining is not altered in respect to normal skin, albeit keratinocytes display a stronger reactivity to anti-bFGF.

Staining of a large panel of tumor tissues revealed a very heterogenous reaction pattern (Table 4). In gastric carcinomas bFGF-bearing cells are nearly exclusively identified as stromal macrophages and only single individual tumor cells stain positively (Figure 2a). This is also true for mammary carcinomas, where besides gland epithelial cells, mainly macrophages contain the growth factor. Further examples of totally negative tumor cells are seen in colon carcinomas (Figure 2b), neuroblastomas (Figure 2c), and adrenal tumors. In these lesions, however, a marked staining reaction is found in endothelial cells surrounding the tumor cells.

In contrast to these negative neoplastic cells, basal and squamous cell carcinomas show an intensive staining reaction that is nearly exclusively confined to tumor cells (Figure 2d). A strong tumor cell reactivity is also seen in osteosarcomas, hemangiomas (Figure 2e), and melanomas; in these staining of the cells seems to depend on their phenotypic differentiation. Primary or metastatic melanoma, for instance, express the antigen predominantly at the tumor invasion front, while tumor cells adjacent to the epidermis are largely devoid of bFGF (Figure 2f). Furthermore, in osteosarcomas bFGF is particularly detected in anaplastic tumor cells while chondroblastic tumor cells are negative. Especially in osteosarcomas and to a smaller extent in melanomas, bFGF is not always located throughout the cytoplasm but seems to be focally accumulated in a perinuclear region (Figure 2f). The identity of this structure is under investigation.

Although most of the analyzed tumor tissues contained considerable amounts of bFGF either in macrophages, endothelial, or tumor cells, there were some especially vascular lesions like granuloma pyogenicum, hemangiosarcoma, and Kaposi sarcoma, or tumors of the ovary and testis, which exhibit only faint or no reactivity to anti-bFGF. It must be noted that in all cell types analyzed bFGF was found to be intracellular rather than in the extracellular matrix. Only in some endothelial cells (Figures 1d, 2e) was expression more pronounced in the abluminal side of the cells, which may be indicative of the presence of bFGF in subendothelial basement membranes.

Discussion

During the last decade, bFGF has emerged as a mediator of major importance for fibrosis and angiogenesis. Several biochemical analyses have revealed the presence of bFGF in a variety of tissues and organs. Limited immunohistologic studies, however, which could provide a more detailed localization of bFGF, have been reported and are confined to heart[20] and muscle tissues[21] or chick embryo[22] and bovine cornea.[23] Thus virtually no information is available on the cell type expressing bFGF *in vivo*. In this study we have analyzed the expression of bFGF in normal, inflammatory, and tumor tissues using affinity-pu-

Table 3. *Distribution of bFGF in Inflammatory Tissues*

Tissues	No. of biopsies	bFGF positive cells
Chronic		
Rheumatoid synovia	5	Synovial cells, macrophages
BCG-granuloma	2	Macrophages, epitheloid cells, few endothelial cells,
Sarcoidosis	2	Macrophages
Liver cirrhosis	3	Hepatocytes, sinusoidal endothelial cells
Endometriosis	5	Macrophages, gland epithelial cells smooth muscle cells
Atherosclerosis (advanced)	5	No staining
Acute		
Psoriasis	3	Keratinocytes
Parodontitis	3	Keratinocytes
Erythrodermia	2	Keratinocytes
Urticaria	2	Keratinocytes

Table 4. *Distribution of bFGF in Tumor Tissues*

Tumor	No. of biopsies	Tumor cells	Macrophages	Endothelial cells
Cutaneous and Vascular Tumors				
Basal cell carcinoma	4	+++	(+)	0
Squamous cell carcinoma	3	+++	0	0
Hemangioma	3	+	0	0
Hemangiosarcoma	1	0	0	0
Granuloma pyogenicum	2	0	0	0
Kaposi sarcoma (classical)	1	0	0	0
Kaposi sarcoma (AIDS)	3	0	0	0
Melanoma	9	++	++	+
Other Tumors				
Gastric carcinoma	5	(+)	+++	0
Neuroblastoma	2	0	0	+++
Adrenal carcinoma	1	0	0	+++
Colon carcinoma	2	0	0	++
Mamma carcinoma	4	(+)	+++	0
Osteosarcoma	2	+++	0	0
Ovary carcinoma	2	0	0	0
Lymph node metastases (melanoma)	2	+	+	+

0 = no reaction at all. (+) = seldom, few single cells. + = weak staining. ++ = moderate staining. +++ = intensive staining.

rified antibodies. A detailed characterization of the antibodies will be presented elsewhere (Risau et al, manuscript in preparation). The staining reaction was clearly specific for bFGF because controls using nonimmune IgG were negative and also adsorption of the polyclonal anti-bFGF antiserum against a recombinant bFGF preparation abolished the staining. However it cannot be excluded that recently described proteins with a high degree of homology to bFGF, such as int-2,[24] K-FGF,[25] FGF-5[26] or FGF-6,[27] crossreact with the antibodies. Of the known numbers of the fibroblast growth factor family, aFGF shares the highest homology (55%) to bFGF but does not show any reaction with the antiserum.

Analyzing several tissues, we found that bFGF expression under normal conditions seems to be restricted to cells of the myeloid lineage. Basic fibroblast growth factor-bearing cells were particularly tissue-fixed macrophages and dendritic cells, which revealed marked staining, especially in thymus and placenta. High levels of staining were also detected in basal keratinocytes of skin biopsies. Keratinocytes may serve as a storage site of bFGF, which may be important in tissue-repair mechanisms. In addition, bFGF has been implicated in autocrine growth control of keratinocytes.[28] In this context it is interesting to note that lesions such as psoriasis, which are characterized by intensive proliferation of keratinocytes, contained higher levels of bFGF than their normal counterparts.

Several groups have shown that *in vitro* cultured endothelial cells can produce bFGF and deposit it partly in their extracellular matrix.[11,29] In our study, the antibody localized bFGF in only a few endothelial cells of normal tissues such as skin or spleen. Staining in endothelial or other cell types was generally found to be cytoplasmic. The intracellular localization seems to be consistent with the absence of a classical signal peptide in the bFGF sequence and inefficient release of the factor. In contrast, other *in vivo* analyses have revealed extracellular matrix-associated bFGF in muscle[21,22] and cornea tissue,[23] while bFGF in heart tissue was determined to be mainly intracellular.[20] On the other hand, we cannot exclude the possibility that small amounts of bFGF are present in the extracellular matrix but are undetectable at the level of sensitivity of the immunohistochemical technique.

In comparison to normal tissues, staining was strongly augmented in inflammatory and neoplastic tissues. The identification of macrophages as the main source of bFGF in these conditions is in accordance with previous findings indicating that the macrophage is the principal effector cell taking part in repair events and tissue homeostasis.[30,31] With respect to angiogenesis, it is notable that other mediators with implications in blood vessel formation as HAF,[32,33] TNF-α,[34,35] or TGF-β[36,37] also were found to be macrophage products.

Staining of inflammatory and tumor tissues was also altered in view of the fact that not only phagocyting cells but also cell types that were unlabeled in normal conditions stained positively. In liver cirrhosis, for example, bFGF was expressed by hepatocytes and sinusoidal cells that were consistently negative in biopsies of unaffected liver. Detailed investigations must show if there is a direct link between bFGF expression and liver fibrosis. In certain other situations, such as parodontitis, acute cutaneous inflammations, or (advanced) atherosclerosis, bFGF was not detectable.

Most striking in inflammatory and tumor tissues was the finding that endothelial-associated staining was strongly enhanced. In some tumors, such as colon carcinoma, neuroblastoma, or adrenal carcinoma, endothelial cells in the vicinity of the tumor were nearly exclusively stained, while other cell types, such as macrophages or tumor cells, were generally devoid of bFGF. The identifi-

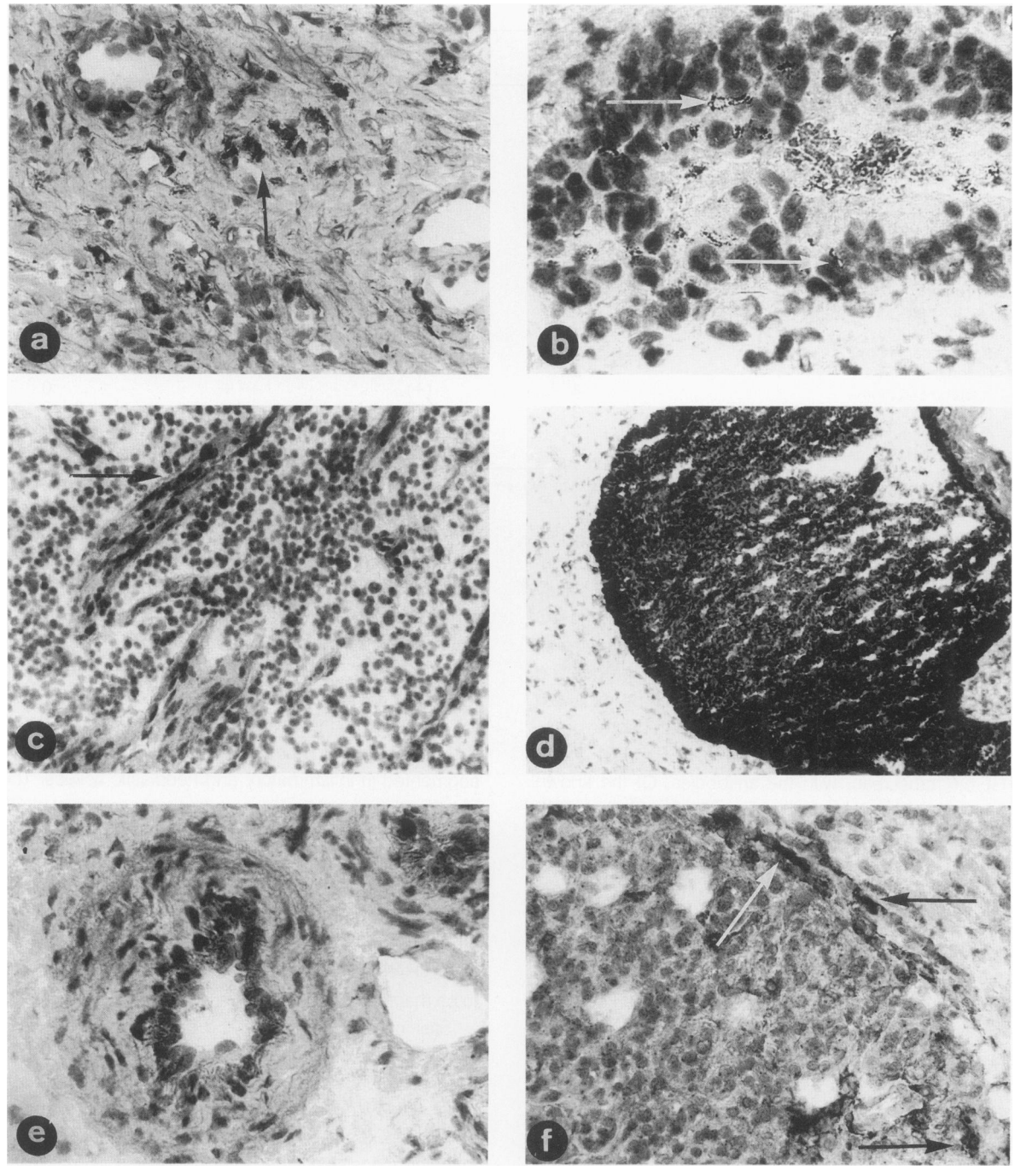

Figure 2. *Detection of bFGF in tumor tissues (frozen sections).* **a:** *Gastric carcinoma: bFGF localizes within infiltrating macrophages (×750).* **b:** *Colon carcinoma: positive capillaries in the vicinity of tumor cells (×1500).* **c:** *Neuroblastoma: positively stained endothelial cells (×600).* **d:** *Basal cell carcinoma: intense bFGF-staining throughout the tumor (×380).* **e:** *Hemangioma: expression of bFGF in vascular cells (×960).* **f:** *Melanoma metastases: positively stained macrophages and tumor cells at the tumor front (×600).*

cation of endothelial cells as a source of bFGF might have implications for understanding the mechanism of tumor angiogenesis, which generally has been thought to be due to angiogenic factors that are released from the tumor cells. In certain tumors, however, the reverse process might be important: tumor cells may release unknown mediators that can stimulate the production of angiogenic factors such as bFGF from endothelial cells. In this case bFGF may act as a self-stimulating growth factor inducing formation of new blood vessels by capillary endothelial cells themselves. Considerations of an autocrine action of bFGF, however, must be tempered by the fact that the mechanism of its release is not understood.

The expression of bFGF in tumors was very heterogeneous, suggesting the involvement of complex mechanisms for tumor angiogenesis. While in some tumors en-

dothelial cells were the prominently positive cell type, in other tumors macrophages or tumor cells represented an accumulation of the growth factor. Infiltrating macrophages in the vicinity of the tumor stained positively in mamma and gastric carcinomas in which individual tumor cells were rarely labeled. In these conditions macrophages may play no role in tumor defense but, in contrast, might contribute to tumor spread. It has been observed in tumors that infiltration of macrophages was correlated to malignancy and poor prognosis.[38,39]

On the other hand, in some tumors such as squamous cell carcinomas or osteosarcomas, bFGF-bearing cells were exclusively tumor cells. Distribution in tumors seemed to be dependent on the functional and differentiation state of the tumor cells. In osteosarcomas anaplastic but not chondroblastic cells revealed bFGF expression. In squamous cell carcinomas, staining reaction was intensified on the outside of the tumor nest. Also melanomas showed a great phenotypic heterogeneity with regard to bFGF expression. While melanomas growing intraepidermally were always negative, bFGF expression tended to increase with the invasion stage of the melanoma. A conspicuous level was especially detected in the invasion front of the tumor. In this context it must be established if bFGF is correlated with a poor cancer prognosis. A contribution of bFGF might be supported by its capability to stimulate increased levels of collagenase and plasminogen activator.[6] An increase in these enzymes may provide the proteolytic activities necessary for the penetration of tumor cells into surrounding tissues.

Therefore tumor-derived bFGF may have pleotropic influences, first on tumor invasion by elevating proteolytic enzymes, and second by paracrine stimulation of endothelial cell growth, which is important for tumor progression and metastases.[40] However this hypothesis should be interpreted with caution because basal cell carcinomas, which always revealed marked levels of bFGF on the one hand, are characterized by an abnormal connective tissue and elevated levels of collagenase[41,42] but, on the other hand, generally reveal rare metastases.

Because it was our principal goal to study the *in vivo* relevance of bFGF in angiogenic events, we can make the following conclusion: bFGF is generally elevated in situations with intensive cellular proliferation and blood vessel formation as tumor growth and inflammation. However, it must be remembered that marked levels of the growth factor can be found in normal tissues with only rare angiogenesis of thymus and skin. Also it is important to note that some tumors, although they revealed marked capillary sprouting (as assessed by the antibody EN 7/44),[43] did not show detectable bFGF. Because the formation of blood vessels is a multistep and multicellular process that is balanced by angiogenic inducers as well as inhibitors,[44,45] it is unlikely that angiogenesis is controlled by a single growth factor.

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